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Running head: TiLV detection.

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ABSTRACT

Tilapia are an important group of farmed fish that serve as a significant protein source, worldwide. In recent years, substantial mortality of wild tilapia has been observed in the Sea of Galilee and in commercial ponds in Israel and Ecuador. We have identified the etiological agent of these mass die-offs as a novel orthomyxo-like virus and named it tilapia lake virus (TiLV). Here we provide conditions for efficient isolation, culturing and quantification of the virus, including the use of susceptible fish cell lines. Moreover, we describe a sensitive nested RT-PCR assay, allowing the rapid detection of TiLV in fish organs. This assay revealed, for the first time, the presence of TiLV in diseased Colombian tilapia, indicating a wider distribution of this emerging pathogen and stressing the risk that TiLV poses for the global tilapia industry. Overall, the described procedures should provide the tilapia aquaculture industry important tools for the detection and containment of this pathogen.

INTRODUCTION

Tilapines, a generic term for edible fish belonging to the family *Cichlidae*, are fast growers, efficient food convertors and relatively disease-resistant. These assets render them most suitable for farming; indeed, tilapines are one of the most significant groups of farmed fish worldwide and serve as an important protein source, especially in developing countries (1-6). Common ectoparasites and the few bacterial pathogens of tilapines are well controlled by pharmacotherapy. Few viral diseases have been reported for tilapia and these are of limited impact (7-9).

Recently, a novel RNA virus termed Tilapia Lake Virus (TiLV) has been identified and recovered from episodes of massive mortalities of wild and pond-cultured tilapia all over Israel (10). High mortalities were also observed in naïve tilapia, exposed to an isolate of TiLV (10). Tilapia mortality, suspected of having a viral etiology, has also been described in Ecuador (11, 12). Although variations in pathological presentation have been described (where lesions were focused in the central nervous system or in the liver, in Israel or Ecuador, respectively), sequencing the whole genome of TiLV

revealed that tilapia in the two countries were infected with almost identical viruses (4). This analysis also revealed that this pathogen is a novel orthomyxo-like virus with a 10-segment, negative-sense RNA genome (4). Segment 1 contains an open reading frame (ORF) with weak sequence homology to the polymerase subunit (PB1) of influenza C virus, while the other nine segments showed no homology to other viruses.

TiLV outbreaks are characterized by high mortalities and economical losses (10, 11) and no vaccines against TiLV are currently available. Thus, there is a great need for the implementation of prompt control measures: culling of infected stocks, setting quarantine, restricting trades and control of possible vectors. This calls for development of fast and sensitive detection methods and improved culturing techniques. Here we show that the presently available reverse transcription (RT)-PCR assay (10), although highly specific, is of limited sensitivity when applied to clinical samples. Accordingly, we now describe a highly sensitive, nested RT-PCR assay for TiLV detection from clinical specimens. In addition, TiLV-sensitive cell lines, other than the reported E-11 cells (10), are described and the optimal parameters for TiLV culturing are defined.

MATERIALS AND METHODS

Cell cultures and infection of cells with TiLV. E-11 (13), TO-2 (14), OmB (15) and TmB (16) cells were grown in Leibovitz (L-15) medium (Gibco, USA), supplemented with 10% inactivated FCS (Gibco), L-glutamine (300 mg/liter), HEPES (pH 7.3; 1%), penicillin (40 U/ml), streptomycin (40 µg/ml), and Nystatin (5 µg/ml). Primary brain cells were prepared and grown as described before (10). For TiLV infections, E-11 monolayers in 25cm^2 flasks (~90% confluence, washed twice with PBS before infection) were incubated with TiLV preparations at 25 °C for 1 h; cells were then washed with PBS and incubated at 25 °C in L-15 medium (supplemented with 10% FCS) and monitored for CPE for up to 14 days. TO-2, OmB and TmB cells were infected with TiLV as described for E-11 cells (see below).

Quantification of temperature-dependent TiLV growth. E-11 cell line and cultures of primary tilapia brain cell (10) (90% confluency in 24-well plates) were infected with TiLV [isolate 4/2011, (10); $10^{3.6}$ $TCID_{50}$/well] and incubated at 15, 20, 25 or 30 °C for up to 19 days. Cells were harvested at the indicated days post-infection and lysed by three freeze-thaw cycles. Total RNA was extracted with peqGOLD Trifast (Peqlab; 30-2010) and levels of TiLV and cellular β-actin RNAs were quantified by quantitative RT-PCR (qRT-PCR). Reverse transcription was performed using Verso 1-step RT-PCR ReddyMix kit (Thermo, Lithuania; AB-1454/LD/A); complemented with primers specified below. Quantification was accomplished by real-time PCR using the ABsolute Blue qPCR SYBR Green Rox Mix (Thermo scientific; AB-4163/A) according the manufacturer's instructions with the following specifications: each reaction contained 3 µl cDNA; TiLV-specific primers [ME1 5'GTTGGGCACAAGGCATCCTA3' and clone 7450/150R/ME2 5'TATCACGTGCGTACTCGTTCAGT3', 300 nM each, amplifying a 250-bp fragment (10)]; annealing and extension were performed at 60 °C for 1 min. To detect β-actin RNA, we used the primers described in (17) (F β-actin 5'GGGTCAGAAAGACAGCTACGTT3' and R β-actin 5'CTCAGCTCGTTGTAGAAGGTGT3', amplifying 143 bp fragment). Continuous fluorescence measurements were achieved with StepOne apparatus (Applied Biosystems). Positive and negative controls consisted of TiLV cDNA and no-template control, respectively. Relative quantification (RQ) was calculated according to (18) with the StepOne software (Applied Biosystems).

Quantification of TiLV growth by end-point dilution assays. E-11, TmB or OmB cell lines were cultured in 96-well plates in 100µl/well of Leibovitz (L-15) medium (Gibco, USA), supplemented with 10% inactivated fetal calf serum (FCS, Gibco, USA). Serial dilutions of TiLV were prepared in the above serum-supplemented medium and 100 µl from each dilution were added to each well (~ 80% confluency). Altogether, 10 wells of each cell line were infected for each dilution. The development of CPE was monitored on a daily basis through 14 days post-infection, when cultures were washed with PBS and stained with crystal violet/formaldehyde/methanol solution. $TCID_{50}$ values were calculated by the method of Reed and Muench (19).

RT- PCR and quantitative PCR (qPCR). To establish a control for building a RT-PCR for detection of TiLV sequences, a 491 bp long PCR fragment, derived from TiLV clone 7450 (GenBank Accession No. KJ605629), was amplified with primers 'Nested ext-1' (5'TATGCAGTACTTTCCCTGCC3') and 'Nested ext-2' (5'TTGCTCTGAGCAAGAGTACC3') (10). The resulting fragment was cloned into pJET1.2/blunt (Thermo Fisher Scientific), which was purified, serially diluted and used (at various known concentrations) as a template for PCR. For these reactions, the following pairs of primers were used: Nested ext-1 and Nested ext-2 (amplifying the 491 bp fragment in a reaction called 'external PCR'); 'ME1' (5'GTTGGGCACAAGGCATCCTA3') and '7450/150R/ME2' (5'TATCACGTGCGTACTCGTTCAGT3') (10), amplifying a 250 bp fragment, embedded in the above sequence (in a reaction called 'internal PCR'); or combination of these two pairs in a nested PCR reaction. For the external or internal PCR reactions (15 µl each), the Verso 1-step RT-PCR ReddyMix kit (Thermo, Lithuania; AB-1454/LD/A) was used with 200 nM (final concentration) of each of the above primers and without the enhancer (DNase). Amplification steps included: 1 cycle of 50 °C, 15 min (to mimic the reverse transcription step); 1 cycle of 95 °C, 2 min; 25 cycles of 95 °C, 60 s / 60°C, 60 s / 72 °C, 60 s and 1 cycle of 72 °C, 7 min. For nested PCR, 3 µl of the external reaction were re-amplified by a PCR reaction (total of 15 µl) of 2X ReddyMix PCR Master Mix (Thermo Scientific; AB-0575/DC/LD/A), using primers ME1 and 7450/150R/ME2 (each at a final concentration of 200 nM). Amplification steps were as above but without the 50 °C, 15 min step. PCR products were separated in a 1% agarose gel by electrophoresis.

The above external and internal PCR reactions of the plasmid dilutions were also quantified by qPCR, using the Fast SYBR Green Master Mix (Applied Biosystems, 4385612) and the cognate set of primers described above (final concentration of 500 nM of each primer, per reaction). To quantify the nested PCR by qPCR, the external PCR reaction was performed with the Verso 1-step RT-PCR ReddyMix kit, as described above; 1 µl of this reaction was then re-amplified with the Fast SYBR Green Master Mix, using primers ME1 and 7450/150R/ME2 (500 nM each). For all qPCR reactions the following steps were used: 1 cycle of 95ºC, 20 s; 40 cycles of 95ºC, 3 s / 60ºC, 30 s. Fluorescence was monitored with StepOnePlus apparatus (Applied Biosystems). Ct values were calculated by the StepOne software.

TiLV RNA detection by nested RT-PCR. Total RNA was extracted from cell cultures, or from liver organs (preserved in RNAlater reagent; QIAGEN; 76104), with EZ-RNA Total RNA Isolation Kit (Biological Industries; 20-400-100) according to the manufacturer's instructions. Reverse transcription and first round (external) PCR were performed using Verso 1-step RT-PCR ReddyMix kit (Thermo, Lithuania; AB-1454/LD/A), essentially according to the manufacturer's instructions but with the following modifications: total volume of the reaction was15 µl, with primers 'Nested ext-1' and 'Nested ext-2' (see above; 200 nM each). Thermal cycling program included: a cDNA synthesis step (50 °C, 15 min); an inactivation step (95 °C, 2 min); a denaturation step (95 °C, 30 s); 25 cycles of annealing (60 °C, 30 s) - extension (72 °C, 1 min); and a final extension step (72 °C, 7 min). 3 µl from the first round PCR were then subjected to re-amplification by a second (nested) PCR of 2X ReddyMix PCR Master Mix (Thermo Scientific; AB-0575/DC/LD/A), essentially according to the manufacturer's instructions but with the following modifications: total volume of the reaction was 15 µl, using primers ME1 and 7450/150R/ME2 (see above; 200 nM each). Thermal cycling program included: an initial denaturation step (95 °C, 2 min); 35 cycles of denaturation (95 °C, 1 min) - annealing (60 °C, 1 min) - extension (72 °C, 1 min); and a final extension step (72 °C, 5 min). PCR products were analyzed by electrophoresis in 1% agarose gels.

Amplification of Nervous Necrosis virus (NNV) RNA. RT-PCR was used to amplify NNV RNA with conditions described above for TiLV RNA, using EZ-RNA Total RNA Isolation Kit and Verso 1-step RT-PCR ReddyMix kit, but with primers F1 (5'GGATTTGGACGTGCGACCAA3') and VR3 (5'TGGATCAGGCAGGAAGC3') and annealing temperature of 54 °C. The length of the amplified product is 254 bp (20).

Processing of clinical samples. Brain samples were collected in Israel between 2011 and 2013, from pond-raised tilapia (*Oreochromis niloticus* x *Oreochromis aureus* hybrids), suspected to have been infected with TiLV. Brains from two ornamental African cichlids, grown in an ornamental fish breeding farm and which showed symptoms of TiLV infection, were also included in this study. Samples from mid-2012 onwards were processed immediately upon arrival; earlier samples were

processed from archived materials (whole fish) stored at -80 ^{0}C. Negative control fish were collected from fish ponds with no apparent disease.

Brains were removed aseptically, pooled (2-3 samples from each outbreak; except from the two samples of ornamental fish that were processed separately) and split into two tubes. The first aliquot was used for RNA extraction and subsequent PCR reactions as described above. The second aliquot was utilized for virus isolation and was manually homogenized with 9 volumes of phosphate-buffered saline (PBS) solution and centrifuged at 3000 x *g* for 10 min; supernatants were filtered through 0.22 µm membrane filters (Starsdet, Germany) and 200 µl were used to infect E-11 monolayers as specified below. For Sample No. 12, E-11 cells that were incubated with brain homogenates and that showed no CPE, were freeze-thawed, and 200 µl of cleared extract was used to infect naïve E-11 cells. This procedure was repeated once more till a clear CPE was observed.

Liver samples, diagnosed histopathologically as having lesions typical of syncytial hepatitis (11), were collected from clinically sick fish, from Ecuador and Columbia. Control livers were collected from unexposed, healthy tilapia (*Oreochromis niloticus*), reared in St. George's University, Grenada. All liver samples were preserved in RNAlater reagent (QIAGEN; 76104).

RESULTS

Temperature-dependent viral growth. Being ectotherms (and cultured at 16-32 ^{0}C range) (21), tilapia potentially may be infected over a relatively wide range of temperatures; yet, the effect of temperature on TiLV replication and thus, on its isolation, has not been studied. Hence, TiLV growth at various temperatures was evaluated by infecting monolayers of E-11 and primary tilapia brain cells, with subsequent incubation at various temperatures (15, 20, 25 and 30 ^{0}C) for up to 19 days. Infection was quantified by qRT-PCR reactions, measuring TiLV RNA expression levels, with TiLV specific primers. Viral RNA levels were normalized to cellular β-actin mRNA levels [Relative Quantification (RQ); Fig. 1 and Materials and Methods]. In E-11 cells, the maximum increase in TiLV RNA levels was observed at

25 ^{0}C at day 9 post-infection (RQ = 1328; Fig. 1A). Higher (30 ^{0}C) or lower (20 ^{0}C) temperatures resulted in reduced TiLV RNA levels (RQ = 191 and 490, respectively, day 9 post-infection). At 15 ^{0}C, TiLV RNA production was dramatically reduced (RQ = 35; day 9 post-infection) and reached maximal levels at day 15 post-infection. In infected primary tilapia brain cells, 25 ^{0}C was also the optimal temperature for TiLV replication (Fig. 1B); yet this replication peaked only at day 14 post-infection and reached much lower levels (about 12%), compared to the one in E-11 cells. Altogether, E-11 cells at 25 ^{0}C provide optimal conditions for TiLV replication, thus all isolations from clinical samples (see below) were carried out under these conditions.

Quantification of TiLV growth in tilapia cell lines. In our former study (10), eight established fish cell lines were tested for their permissiveness to TiLV infection and only E-11 cells were found suitable for this purpose. We now extended this analysis to three additional tilapia cell lines, derived from ovary [TO-2; (14)], brain [OmB; (15)] and bulbus arteriosus [TmB; (16)]. Initial qualitative analyses revealed that OmB and TmB, but not TO-2, support TiLV replication (data not shown). We next compared the use of the permissive cell lines (E-11, OmB and TmB) in the quantification of TiLV infection by endpoint dilution assays. The cell lines were infected with dilutions of the virus, CPE was monitored for 14 days and $TCID_{50}$ values were calculated. The results of three independent experiments are shown in Table 1. All three cell lines showed comparable sensitivities to TiLV infection. Yet, E-11 cultures were superior because the CPE development was clearly detected in a relative short time (~4, 6 or 8 days post-infection for E-11, TmB or OmB, respectively). OmB cells also provided convenient way to monitor CPE at longer time post-infection (~14 days), as uninfected cells remained attached as monolayers while infected cultures completely detached at this time point. TmB cells were sensitive to TiLV-induced CPE, yet we found that detecting CPE in this line was more difficult compared to the other lines because TmB cells did not support the formation of clear plaques and only a portion of the infected cells detached from the plate over time.

Sensitivity and specificity of TiLV detection by PCR. A sensitive RT-PCR detection method for TiLV is required for rapid and accurate diagnosis of this threat

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to tilapine aquaculture. We have described TiLV detection by RT-PCR (10); however, assays were not optimized. To optimize this procedure, we first prepared a standard curve for this assay. Specifically, a 491 bp long PCR fragment, derived from TiLV clone 7450 (GenBank Accession No. KJ605629) (10), was cloned into a plasmid and dilutions of the resulting plasmid DNA were used in the following PCR/gel electrophoresis (Fig. 2A) and qPCR (Fig. 2B) reactions. Different sets of primers were used to amplify either the 491 bp fragment ('external PCR'; Fig. 2A, Upper Panel) or an internal 250 bp fragment ('internal PCR'; Fig. 2A, Middle Panel). Additional reaction ('nested PCR'; Fig. 2A, Lower Panel) consisted of the external PCR, combined with the internal PCR (Materials and Methods). This analysis showed that as expected, the external and internal PCR reactions were less sensitive than the nested PCR: the highest dilution in which the TiLV sequence was clearly detected by the external or the internal PCR reactions was 10^{-6} (Fig. 2A, Lane 2, Upper and Middle Panels); relating to detection of ~70,000 TiLV copies. The nested PCR showed much higher sensitivity, enabling the detection of as low as 7 copies of TiLV sequence (Fig. 2A, Lane 6, Lower Panel). Amplification of the above diluted plasmid DNA by qPCR also demonstrated the higher sensitivity of the nested PCR over the non-nested PCR reactions as much lower threshold cycle (Ct) values were obtained for the former reaction (Fig. 2B). Of note, the detection limit of the nested qPCR (70 copies, Fig. 2B), was higher than that of the nested PCR (7 copies, Fig. 2A). These differences likely result from the different reagents and conditions used for these two types of reactions (Materials and Methods).

The specificity of the developed nested PCR was further demonstrated by the amplification of TiLV sequences from cDNAs that were prepared from TiLV-infected E-11 cells; but not from negative samples composed of cDNAs of NNV-infected, or naïve E-11 cells (Fig. 2C).

TiLV detection in diseased tilapia from Israel. Based on the optimal conditions for TiLV growth and detection defined above, we next set out to isolate TiLV from clinical specimens obtained from 13 different outbreaks between 2011 and 2013 in nine different commercial farms, distributed over Israel (Galilee, Jordan valley and Mediterranean coastal areas). In all these outbreaks, diseased fish showed typical symptoms related to TiLV infection (10)**.** Brain samples were obtained from

commercial pond-raised tilapia for human consumption (*Oreochromis niloticus* x *Oreochromis aureus* hybrids; Specimens No. 1-11, Table 2), and ornamental African cichlids (Specimens No. 12 and 13, Table 2). Brain was chosen for these analyses because this tissue is relatively confined and susceptible to TiLV infection (10). The brains were homogenized (pools of 2-3 brains for each outbreak for Samples No. 1-11; Samples No. 12 and 13, each consisted of a single brain) and added to E-11 cells, cultured at 25 ^{0}C. This procedure resulted in the appearance of CPE at 5 to 6 days post-inoculation, in 12 out of the 13 cases (Table 2). For Specimen No. 12, two additional passages in E-11 cell cultures were required before CPE became apparent (Materials and Methods). No CPE was observed for a negative control group, consisting of 15 fish that were collected from ponds showing mortalities due to either environmental conditions (low oxygen levels or high ammonia concentrations) or other infectious diseases (i.e. streptococci) (data not shown).

The above 13 brain tissues were also tested for the presence of TiLV sequences by the internal and nested PCR reactions described above. For this, total RNA was extracted from portions of the brains, reverse transcribed using random primers and PCR amplified with TiLV-specific primers (Materials and Methods). The internal PCR detected TiLV sequences in only three samples (23%), in contrast to the nested PCR that detected the virus in 12 samples (92%, Table 2). The amplification of TiLV sequences was also verified by sequencing the PCR products (data not shown). None of the negative controls scored positive when examined by the nested PCR, further demonstrating the specificity of this assay (data not shown).

TiLV detection in diseased tilapia from South America. To further examine the developed nested RT-PCR, we applied it to detect TiLV RNA in liver samples, preserved in RNAlater reagent, that were taken from South American tilapia, showing signs of syncytial hepatitis (11, 12), or from healthy controls. This test was performed in a blinded way using the following procedure: the presence or absence of TiLV RNA in the samples was tested by RT-PCR (12), in St. George's University, Grenada. The samples were then coded and shipped, preserved in an RNAlater reagent, to Tel Aviv University, where RNA was extracted and nested RT-PCR was performed without knowing the samples' identities. Fig. 3A shows the results of this procedure for Ecuadorian samples: six examined samples scored positive (Lanes 1-6) while six

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samples scored negative (Lanes 7-12). This fully matched the classification made of the samples, before shipment.

Tilapia with syncytial hepatitis were also observed in farms in Colombia and liver samples were examined for the presence of TiLV RNA, as above. This analysis revealed that out of the six samples that were scored positive for TiLV, four samples also scored positive after their shipment (Fig. 3B, Lanes 1-4), while the two other samples scored negative (Fig. 3B, Lanes 5-6). This discrepancy likely resulted from the degradation of TiLV RNA in these samples. Indeed, attempts to amplify TiLV RNA from these two samples using different sets of primers that were derived from another segment of TiLV genome, failed too (data not shown). For the negative samples, no PCR products were observed (Fig 3B, Lanes 7-12).

Overall, these results demonstrate that the developed nested RT-PCR can be applied for detection of TiLV strains in Israel and South America and suggest that preserved material can be analyzed too. Importantly, these results further show for the first time that TiLV is present also in tilapia farmed in Colombia, and confirm the global distribution of this newly recognized pathogen.

DISCUSSION

TiLV, a recently identified pathogen, causes recurrent outbreaks in wild and cultured tilapia. These outbreaks are characterized by significant mortality and morbidity, resulting in massive losses to tilapia industry both in Israel and South America (4, 10-12). Thus, efficient methods for TiLV isolation and detection are required.

Temperature is the first parameter that we examined for optimization of TiLV culturing, since outbreaks of viral diseases of fish are typically temperature-dependent (22). Of note, the temperature at which a disease occurs does not necessarily reflect the optimal temperature for the *in vitro* growth of the cognate pathogen. For example, deadly outbreaks of viral hemorrhagic septicemia (VHS) in farmed Japanese flounder, caused by viral hemorrhagic septicemia virus (VHSV), occurred when water temperatures were between 8 and 15 °C, while the isolated VHSV strain replicated most rapidly at 20 °C (23). Similarly, spring viremia of carp (SVC), caused by

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spring viremia of carp virus (SVCV), occurs with high mortalities at water temperatures of 10 to 17 °C, while the optimum temperature for the *in vitro* replication of SVCV is 20 °C (24). In the case of TiLV, the broad range of water temperature (~ 24 to 33 °C) that occurs during the hot season (May to October) (10), calls for determination of the optimal temperature for efficient virus growth, *in vitro*. Our results (Fig. 1) clearly demonstrate that 25 ^{0}C allows maximal growth of TiLV.

We also determined TiLV growth in several types of fish cells. When comparing primary tilapia brain cells to E-11 cells, TiLV replication generated much higher viral RNA in the latter cells, despite the fact that E-11 cells are derived from the snakehead fish (*Ophicephalus striatus*) (13, 25) - a freshwater perciform fish (family *Channidae*), which is distant from tilapines (family *Cichlidae*). Our present study identified two additional tilapia cell lines that support TiLV growth: the OmB; (15) or TmB (16) cells, derived from tilapia brain or bulbus arteriosus, respectively. In respect to CPE development, E-11 and OmB were superior compared to TmB. Plaques where readily detected in E-11 cells, whereas TiLV-infected OmB cultures were characterized by almost complete detachment from the plate. Thus, E-11 cells are convenient for plaque assays and OmB cultures are useful in end-point dilution ($TCID_{50}$) assays. E-11 cells, which are derived from a species distant from the natural host, should also be useful in studies involving TiLV attenuation. Yet, E-11 cells also produce the snakehead retrovirus (SnRV) (13) and this may hamper the development of pure vaccine strains for TiLV. Since OmB and TmB cells are SnRV-free (our unpublished results), these cells should be useful in generating pure TiLV strains.

We demonstrated that TiLV culturing is a sensitive method for detection of the virus. Yet, this methodology is time consuming and labor intensive; thus, it is inadequate when prompt and accurate control measures are required (i.e. culling of infected stocks). Hence, we developed RT-PCR-based techniques that are fast and sensitive. We demonstrated that the nested RT-PCR protocol, described here, detects only few molecules of TiLV genome and can be applied in detecting TiLV RNA in fresh and preserved organs of diseased fish. The protocol is based on amplification of consensus regions that were identified by analyzing high-throughput sequencing data, obtained from TiLV samples collected in Israel and Ecuador. This analysis revealed high sequence homology between the Israeli and Ecuadorian samples across TiLV genome

(4) and thus, all TiLV segments can be used as templates in RT-PCR reactions. The four primers used in our protocol are derived from Segment 3 of the Israeli isolate of TiLV (4, 10). Three primers (Nested ext-1, Nested ext-2 and ME1) fully match sequences of TiLV obtained from 12 Ecuadorian samples. The fourth primer (7450/150R/ME2) fully matches eight of the 12 Ecuadorian samples, but has a single mismatch in its second position, compared to the other four samples (sequences of the latter contain a G instead of an A). This 5' mismatch should not interfere with amplification and the described set of primers readily amplified TiLV sequences from samples obtained from disease outbreaks in both Israel and Ecuador. Moreover, the power of this RT-PCR-based assay was exemplified when it detected TiLV in organs of diseased tilapia, obtained from yet another country - Colombia.

This is the first report of TiLV occurrence in Colombian aquaculture, which adds to the reports of TiLV outbreaks in Israel and Ecuador. This substantiates TiLV as an emerging pathogen and highlights the risk that TiLV poses for the global tilapia industry. The methods described here should detect the virus through early onset of TiLV infection, assisting its containment.

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E.B., A.E., N.M., T.B., and W.I.L. have applied for patents in the fields of TiLV diagnostics and vaccines.

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FIGURE LEGENDS

Figure 1. TiLV replication at different temperatures. E-11 (A) and primary brain (B) cultures in 24-well plates were infected with TiLV and incubated at the indicated temperatures. Total RNA was extracted from infected cells at the indicated time points postinfection and TiLV and cellular β-actin RNA levels were quantified by qRT-PCR. The results show the relative quantification (RQ) of TiLV RNA levels, relative to β-actin RNA levels (means of duplicates ± standard deviations).

Figure 2. Sensitivity and specificity of PCR, nested PCR and qPCR in the amplification of TiLV. (A) Ten-fold dilutions of a plasmid containing a 491 bp long PCR fragment, derived from TiLV clone 7450 (GenBank Accession No. KJ605629), were subjected to PCR with primers, amplifying either a 491 bp (Upper Panel) or 250 bp (Middle Panel) fragments. A nested PCR, using the above two sets of primers was also performed (Lower Panel). Lanes 1 to 9 show the PCR products for 10^{-5} to 10^{-13} dilutions range, respectively, separated on a 1% agarose gel by electrophoresis. The PCR reaction that amplified the 10^{-10} dilution (Lane 6) contained 7 copies of TiLV sequence. (B) qPCR reactions were also applied to the dilutions and primer pairs described in (A). The values of the threshold cycle (Ct) were plotted against calculated TiLV copies and trendlines were added using Excel software. Reactions were run in triplicates and only the linear range is shown. (C) cDNAs of TiLV-infected E-11 cells (Lane 1), Naïve E-11 cells (Lane 2) or NNV-infected E-11 cells (Lane 3) were subjected to Nested PCR with TiLV-specific primers as in (A), to detect TiLV sequences. Amplification of TiLV sequences from the plasmid described in (A) was used as a positive control (Lane 4). Amplification reaction with no DNA template served as a negative control (Lane 5). The absence or presence of NNV sequences in cDNAs, prepared from naïve (Lane 6) or NNV-infected (Lane 7) E-11 cells, respectively, was confirmed by PCR with NNV-specific primers. M, denotes size markers.

Figure 3. Detection of TiLV RNA in preserved tilapia livers from Ecuador and Columbia. Nested RT-PCR was used to determine the presence or absence of TiLV RNA in liver samples, preserved in RNAlater reagent. (A) Samples from Ecuador of diseased (Lanes 1-6) or healthy fish (Lanes 7-12). Reaction with no RNA served as a

533 negative control (Lane 13). (B) Samples from Columbia of diseased (Lanes 1-6) or
534 healthy fish (Lanes 7-12). M marks DNA size markers.
535
536 TABLES
537
538 **TABLE 1. [a]Comparison of $TCID_{50}$ values for three TiLV-susceptible cell lines**

	Cell Lines		
Experiment No.	E-11	TmB	OmB
1	$3.2x10^5$	$5x10^5$	$1.6x10^5$
2	$4x10^6$	$4x10^5$	$5x10^5$
3	$1.6x10^5$	$2x10^5$	$1.6x10^5$

539 [a]The same stock of TiLV (grown in E-11 cells) was quantified in three independent
540 endpoint dilution assays. Values are in $TCID_{50}$/ml.
541
542 **TABLE 2. TiLV detection in clinical specimens by culturing, RT-PCR and**
543 **nested RT-PCR.**

Specimen No.	Location	[a]CPE	RT-PCR	Nested RT-PCR
1	Farm 1, Galilee	+	+	+
2	Farm 1, Galilee	+	-	+
3	Farm 2, Jordan Valley	+	-	+
4	Farm 2, Jordan Valley	+	-	+
5	Farm 3, Jordan Valley	+	+	+
6	Farm 4, Jordan Valley	+	+	+
7	Farm 4, Jordan Valley	+	-	+
8	Farm 5, Jordan Valley	+	-	+
9	Farm 6, Coastal	+	-	+

	region			
10	Farm 7, Jordan Valley	+	-	+
11	Farm 8, Galilee	+	-	-
12	Farm 9, Jordan Valley	[b]+	-	+
13	Farm 9, Jordan Valley	+	-	+
% Positives		100	23	92

544 [a]CPE was detected in E-11 cells, incubated with the brain specimens.

545 [b]CPE visible only after two additional passages on E-11 cell cultures.

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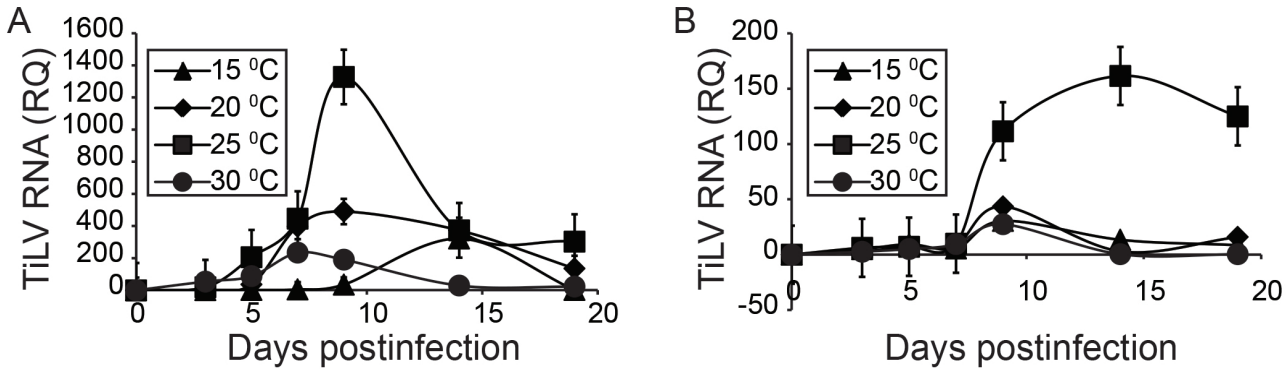

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